The mature *AEP2* gene product of *Saccharomyces cerevisiae*, required for the expression of subunit 9 of ATP synthase, is a 58 kDa mitochondrial protein

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Abstract **The nucleotide sequence of the yeast nuclear *AEP2* gene, required for the expression of the mitochondrial DNA-encoded subunit 9 of ATP synthase, predicts a primary translation product of 67.5 kDa. The *ATP13* gene is allelic to *AEP2* but was reported to encode a protein of about 42 kDa in size. We thus investigated genetically and biochemically the size of the *AEP2* gene product. Genetic complementation assays using 3′ truncated *AEP2* genes, here shows that function is abolished by the removal of only 32 amino acids from the C-terminus of the predicted protein product. Cell-free translation of *AEP2* produces a 64 kDa polypeptide (consistent with the *AEP2* sequence) which is imported into mitochondria and processed to a 58 kDa product by the removal of a presequence of about 50 amino acids.**

Key words: *AEP2* gene; Mitochondrial ATPase complex; Import; Nuclear–mitochondrial interaction; Translation, in vitro; *Saccharomyces cerevisiae*

1. Introduction

The oligomeric mitochondrial F_1F_O-ATP synthase complex (mtATPase) of *Saccharomyces cerevisiae* is comprised of at least 11 polypeptides divided into two sectors [1]. The hydrophobic F_O sector forms a proton-translocating channel across the inner membrane of the organelle. The hydrophilic F_1 sector possesses ATP synthase/hydrolase activity and protrudes into the mitochondrial matrix. Structural and functional interactions of the F_O and F_1 sectors are mediated by a group of proteins in the interconnecting stalk [2]. The F_O sector of yeast mtATPase comprises three membrane-associated subunits, 6, 8 and 9, that are encoded by the mitochondrial genome and are translated on mitochondrial ribosomes prior to becoming assembled into the inner membrane of the organelle. In contrast, all subunits of the F_1 sector and the stalk are encoded by nuclear genes, translated on free cytosolic ribosomes and translocated post-translationally into the mitochondrial matrix for assembly into the complex [1].

Aside from those genes that directly encode mtATPase subunits, the identification of nuclear genes necessary for the formation of a functional mtATPase complex [3–10] has emphasised the processes underlying the coordinate expression of both nuclear and mitochondrially encoded subunits. These investigations have been aided greatly by the use of yeast strains deficient in respiratory function due to nuclear mutation. Using such mutants, two independent studies have reported the isolation and characterisation of functional nuclear DNA sequences required for the expression of the mitochondrial *oli1* gene encoding mtATPase subunit 9 [5,8]. Mutations in these nuclear sequences have been shown to cause the abolishment of subunit 9 synthesis [10,11] associated with a decrease in abundance of the mature *oli1* mRNA [5,10]. The persistence of precursors to the *oli1* mRNA in the mutants led to the hypothesis that the mutable function is required for the maturation or translational stability of the *oli1* mRNA [5,10]. In our laboratory, a gene designated *AEP2* was identified on a DNA segment encoding an open reading frame (ORF) predicting a protein product of 67.5 kDa in size [8]. In a parallel investigation by Ackerman et al. [5], a gene designated *ATP13* was identified. A protein product of about 42 kDa was observed after translation in vitro, which was demonstrated to be imported into isolated yeast mitochondria [5]. This observed polypeptide size was consistent with the originally reported sequence of *ATP13* (predicting a 42.9 kDa product). The *ATP13* sequence [5] was almost identical to that of *AEP2* [8], indicating an allelic relationship. The substantial size difference between the predicted products of these alleles arises from the presence of a thymidine residue (T^{1087}) in the *AEP2* sequence that is absent from that of *ATP13*. The resulting frameshift causes the *AEP2* ORF to be 208 codons longer than that of *ATP13*.

Recently, it has come to our attention (A. Tzagoloff and S.H. Ackerman, personal communications) that the published sequence of *ATP13* [5] contains several sequencing errors, the most critical of which led to the missing nucleotide at position 1087. Since the protein products predicted from the ORFs of both *AEP2* and *ATP13* now are the same size (67.5 kDa), it is important to establish independently the size of the protein product (Aep2p) of the *AEP2* gene. To provide a sound basis for our ongoing studies on the involvement of *AEP2* in *oli1* expression, we have examined Aep2p genetically and biochemically. The results are consistent with the *AEP2* ORF encoding a protein of about 64 kDa (close to that predicted from the DNA sequence), which is demonstrated to be readily imported into mitochondria.

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Abbreviations: mtATPase, mitochondrial proton-translocating ATPase (ATP synthase); Aep2p, protein product of the *AEP2* gene; ORF, open reading frame; PCR, polymerase chain reaction; kb, kilobase pairs; bp, base pairs; kDa, kilodaltons.

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2. Materials and methods

2.1. Yeast strains and growth

Strains of *S. cerevisiae* used in this study are as follows: SC167 *MATα ade1* [*rho*$^+$] [10]; OL-1 *MATα his3–11,3–15 leu2–3,2–112 ura3–251,3–373* [*rho*$^+$] [12]; J69–1B *MATα ade1 his6* [*rho*$^+$][13]; DC5 *MATa can1–11 his3 leu2–3,112* [*rho*$^+$] [14]; W4–2A *MATα ade1* [*rho*$^+$] [9]; MP3–8C *MATa ade1 leu2 aep2*^{ts} [*rho*$^+$] [10]. Growth media and culture conditions were described previously [8].

2.2. Primers and plasmid constructs

The oligonucleotide primers used in this study were TE1 (5′-CATTAATCATGGATCCA GGTAGTGGAC-3′), TE2 (5′-ATTCTACTTACAGGATCCTTACCAAAGGC-3′), PF3 (5′-GCTGATCTTGCTCTGCAGGAG-3′) and PF4 (5′-ATGACTTAGCTGAAACAGCC-3′). Standard procedures were used to produce and manipulate recombinant plasmids and bacteriophage [15]. The construction of plasmid pMP35 (Fig. 1) has been described previously [8]. The inserts for pMF10, pMF12, pMF13 and pMF15 (Fig. 1) were obtained from deletion clones constructed by partial exonuclease III digestion of the entire pMP35 insert (a *Bam*HI/*Xba*I chromosomal DNA fragment). After *Xba*I and *Kpn*I digestion and 3′-to-5′ exonucleolytic digestion from the *Xba*I site, pMP35 was treated with S1 Nuclease (Promega, Australia). The ends of the treated plasmid were repaired using the Klenow Fragment (of DNA polymerase I) (Pharmacia, Australia) and ligated together. During the deletion reaction, the *Xba*I site was destroyed, so the deletion products were excised from the M13mp18 vector as *Bam*HI/*Bgl*II fragments. The deletion-carrying fragments were ligated into the *Bam*HI site of the 2 μm-based yeast/*Escherichia coli* shuttle vector YEp351 which contains the yeast *LEU2* gene as the selectable marker in yeast [16].

2.3. In vitro gene expression and protein import

For in vitro production of the *AEP2* product, primers TE1 and TE2 were used, in conjunction with the Muta-Gene in vitro mutagenesis kit (Bio-Rad, Australia), to introduce *Bam*HI cleavage sites 12 bp upstream and 1 bp downstream of the *AEP2* ORF [17]. The resulting *AEP2* cassette was ligated in the sense orientation into the *Bgl*II site of pSP64T [18]. Transcription and translation in vitro and assays of yeast mitochondrial import of the radiolabelled protein product were done as described [13,18] using mitochondria isolated from strain J69-1B.

2.4. Polymerase chain reaction and sequencing

Amplification of DNA fragments using the polymerase chain reaction (PCR) was done in 100 μl reactions containing 10 mM Tris-HCl, pH 8.5, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dGTP and dTTP, 0.2 μM each primer PF3 and TE2, one unit *Taq* DNA polymerase (Perkin Elmer, Australia) and various dilutions of total yeast DNA. The reactions were heated 95°C for 5 min and then incubated in a thermocycler (Hybaid, UK) for 30 cycles. The parameters for each cycle were 95°C for 2 min, 45°C for 2 min and 72°C for 2 min. The PCR products were purified through Sephadex G-50 (Pharmacia, Australia) spin-columns [15] and precipitated with ethanol. Single-stranded template for sequencing was produced using the same conditions as for PCR except 2 ng of purified double-stranded PCR product was used as template and 20 pmol of either PF3 or PF4 was used as the sole primer. The products were extracted with chloroform/phenol/isoamyl alcohol (25:24:1), precipitated with ethanol and sequenced directly with either PF3 or PF4 by the dideoxynucleotide chain termination method [19] using a Deaza ^{T7}Sequencing kit (Pharmacia, Australia).

2.5. Miscellaneous methods

Standard methods were used to introduce plasmids into strains of *E. coli* [15] and yeast [20]. Total yeast DNA was prepared using a published method [21].

3. Results and discussion

3.1. Presence of T^{1087} in the AEP2 gene of laboratory yeast strains

In view of the earlier difficulties regarding the presence or absence of nucleotide T^{1087} in *ATP13*, we initially sought to confirm the presence of that critical nucleotide amongst several laboratory strains of yeast from widely different sources. Using primers PF3 and TE2 (Fig. 1), an approximately 850 bp fragment was amplified by PCR directly from the total genomic DNA of these five presumably unrelated haploid strains (listed in section 2). Direct sequencing of both product strands revealed that all five strains contained T^{1087}, demonstrating that only *AEP2* is found in these strains. This analysis included strain W4-2A which was the source of DNA for the genomic library from which *AEP2* was originally isolated [8]. Additionally, T^{1087} was also found during the sequence analysis of the *aep2*^{ts} allele from the SC167-derivative strain MP3-8C, and is thus unrelated to the temperature-sensitive phenotype of this mutant.

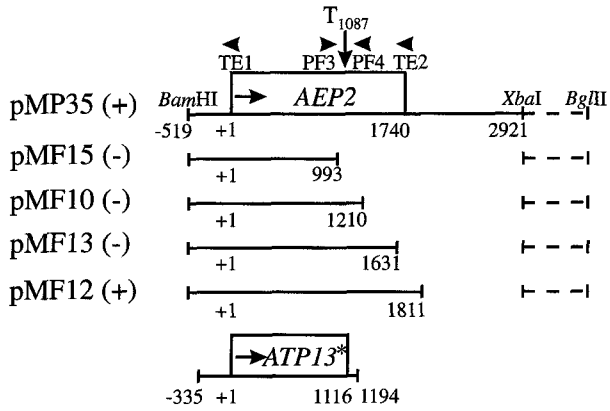

Fig. 1. Segments of yeast chromosomal DNA derived from the *AEP2* region. The plasmid inserts used in this study (propagated in the vector YEp351) and the relative locations of the *AEP2* [8] and the originally published *ATP13** [13] sequences are shown diagrammatically. The location of T^{1087} (a nucleotide present in *AEP2* but not initially reported in *ATP13**) is indicated (vertical arrow). Note that the *ATP13** sequence has since been revised to indicate the presence of T^{1087} in *ATP13* (see text). ORFs (boxes) and the direction of transcription (horizontal arrows) are indicated. Numbers below the horizontal lines are nucleotide coordinates relative to the first nucleotide of the ATG initiation codon of *AEP2* at position +1. The ability (+) or inability (−) of each plasmid to rescue phenotypically the *aep2*^{ts} mutant MP3-8C is shown. Arrowheads mark the location and direction (5′-to-3′) of primers TE1, TE2, PF3 and PF4. In the deletion clones pMF12, pMF13, pMF10 and pMF15 derived from pMP35, gaps indicate the deleted sequences; the region from the *Bgl*II site to the deletion end-point (650 bp) is derived from the vector M13mp18 (dashed line).

3.2. Functional assessment of AEP2 coding region

It is important to establish if the full-length of the coding region of the *AEP2* sequence is required for function, in relation to the reported size of the *ATP13* product as 42 kDa. Our attention focussed on the 3′ end of the *AEP2* DNA segment, in order to confirm that the coding region downstream of the nucleotide T^{1087} was indeed essential for the function of Aep2p.

A genetic complementation assay employing the temperature-conditional *aep2*^{ts} mutant MP3-8C was used to determine if truncated versions of *AEP2* were functionally active. At the permissive temperature of 22°C, MP3-8C is able to utilise the non-fermentable substrate ethanol as the sole carbon source (Fig. 2B), indicative of functional oxidative phosphorylation, and hence mtATPase function. However, at the restrictive tem-

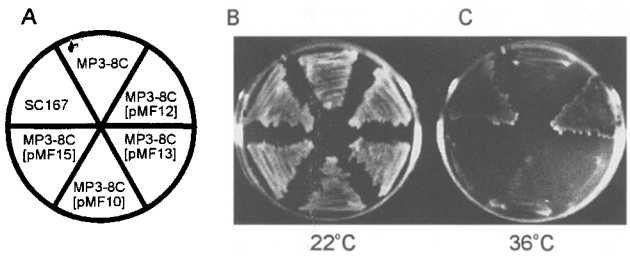

Fig. 2. Plasmid-mediated rescue of the temperature-conditional *aep2*^{ts} mutant MP3-8C by truncated forms of *AEP2*. Yeast strain MP3-8C, either untransformed or transformed with pMF12, pMF13, pMF10 or pMF15, and strain SC167 were streaked in an array (panel A) on nutrient plates (rich medium) containing the non-fermentable substrate ethanol. Plates were incubated four days at either 22°C (panel B) or 36°C (panel C).

perature of 36°C, MP3-8C is unable to utilise ethanol (Fig. 2C) due to the lack of a functional mtATPase, as a result of the *aep2*^{ts} mutation [10]. The MP3-8C wild-type parental strain SC167 is able to utilise ethanol at either temperature (Fig. 2B and C). As previously shown [8] strain MP3-8C transformed with the full-length *AEP2* ORF in pMP35 grows as well on ethanol-containing media at 36°C as does the parental strain SC167 due to functional complementation of the *aep2*^{ts} mutation by the plasmid-borne copy of *AEP2*.

To determine if the C-terminal 208 amino acid extension of *AEP2* beyond that encoded by the *ATP13* ORF is necessary for function, a series of transformants of MP3–8C was prepared, using plasmids (Fig. 1) bearing deleted versions of the pMP35 insert. The transforming plasmids contain, in each case, a progressively longer deletion that truncates the *AEP2* ORF closer to the initiation codon. Strikingly, all three deletions (pMF15, pMF10 and pMF13) that truncate the *AEP2* sequence within the ORF, including two where the truncation point is downstream of the critical residue T^{1087}, fail to rescue (Fig. 2C). Note that the predicted size of the non-functional C-terminally truncated Aep2p product of the pMF13 construct is minimally 60 kDa, much larger than 42 kDa. Only the plasmid with the shortest deletion, pMF12, in which the truncation of the *AEP2* region takes place 70 bp downstream of the *AEP2* ORF, functionally complements the *aep2*^{ts} mutation in MP3-8C (Fig. 2C). The phenotypic rescue of MP3-8C by pMF12 is indeed plasmid-mediated: when the transformants were grown under non-selective conditions, loss of the plasmid (in Leu$^-$ segregants) was always accompanied by loss of the ability of cells to grow on ethanol medium at 36°C (data not shown).

The failure of shorter truncated segments of the pMP35 insert to complement the *aep2*^{ts} mutation confirms the *AEP2* ORF as a functional unit and indicates that the 208-residue C-terminal region of the *AEP2* ORF encoded downstream of T^{1087} is required for activity, at least in the strains studied here.

3.3. *In vitro translation of the Aep2p protein and its import into isolated mitochondria*

In order to investigate the size of the protein encoded by the *AEP2* ORF, and its ability to be imported into mitochondria, the *AEP2* ORF as a *Bam*HI cassette was subcloned into the pSP64T vector suitable for in vitro transcription and translation [18]. The radiolabelled translation product (Fig. 3, lane 1) contains several prominent species; the largest, at 64 kDa, is somewhat smaller than the 67.5 kDa predicted for Aep2p. This minor discrepancy may arise from a structural feature of Aep2p that causes it to diverge from the size/mobility relationship followed by the reference proteins used as size standards. From the sizes estimated for the multiple shorter translation products and the distribution of methionine in the putative protein product of the *AEP2* ORF, it appears the shorter products could arise from non-stringent selection of initiating AUG codons by the rabbit reticulocyte lysate, resulting in initiation of translation at many AUG codons.

Incubation of the translation products with active mitochondria under conditions suitable for protein import, followed by re-isolation and washing of the organelles, resulted in the binding to mitochondria of many of the species present in the translation product (Fig. 3, lanes 2, 4, 6, 8 and 10). This binding reaction was virtually complete within 5 min (Fig. 3, lane 4) as further incubation did not enhance the amount of radiolabel associated with the particulate fraction (Fig. 3, lanes 6, 8 and 10). Relatively little radiolabelled material became associated with mitochondria after only a very brief exposure of the organelles to the translation product (Fig. 3, lane 2). When proteinase K was added to the import mixtures, a portion of the bound 58 kDa polypeptide was found to be the only resistant species (Fig. 3, lanes 5, 7, 9 and 11), indicating import of this polypeptide into a compartment that is inaccessible to externally added protease. Unlike precursor binding, the import of the 58 kDa polypeptide was apparently incomplete after 5 min (Fig. 3, lane 5), but was essentially complete by 10 min (Fig. 3, lane 7). The kinetics of binding and import thus suggest that these processes occur sequentially and independently.

When mitochondria inactivated by the collapse of the electrochemical potential were incubated together with the radiolabelled precursor for 30 min (Fig. 3, lane 12), no polypeptides were found to be resistant to proteinase K digestion (Fig. 3, lane 13). This establishes that the acquisition of protease resistance is an energy-dependent process, and thus conforms to the features expected of a mitochondrial import reaction [18]. The complete proteinase K susceptibility of the radiolabelled precursor incubated in the presence of either active mitochondria for 0 min (Fig. 3, lane 3) or inactivated mitochondria for 30 min (Fig. 3, lane 13) excludes the possibility that the proteinase

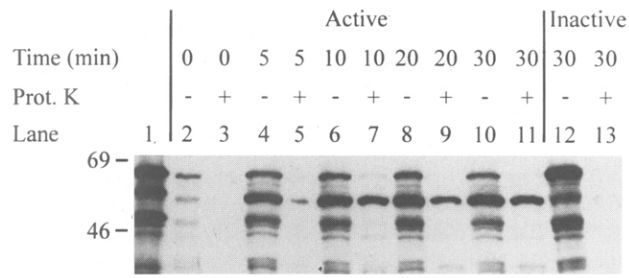

Fig. 3. Import of Aep2p into isolated mitochondria. Radiolabelled *AEP2* translation product (lane 1) was incubated for the times (min), indicated on the figure with active (lanes 2–11) or inactive (lanes 12 and 13) mitochondria isolated from strain J69-1B. After re-isolation of the mitochondria from the incubation mix, one-half of each import reaction was left untreated (−) while the remainder was digested with proteinase K (+). An equal portion of each sample was then separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis on a 7.5% gel [13]. The locations of molecular weight markers, in kDa, are shown on the left.

K-resistant portion of the 58 kDa species found in the reactions using active mitochondria is due to incomplete digestion by proteinase K.

Of the multiple polypeptides among the radiolabelled translation products (Fig. 3, lane 1), in principle only the 64 and 59 kDa species could give rise to the 58 kDa proteinase K-resistant species. However, in the absence of import (Fig. 3, lane 12), the 64 kDa polypeptide is the predominant species to become bound to added mitochondria. This experiment suggests that only the 64 kDa species binds to the mitochondria and undergoes processing during import to become the 58 kDa polypeptide.

3.4. *The protein product of* AEP2 *and its mitochondrial location*

The N-terminus of Aep2p (in common with that of the *ATP13* product) is positively charged and rich in hydroxylated amino acids which is characteristic of typical mitochondrial targeting sequences [22]. We therefore consider the 64 kDa product to be the most likely candidate for the direct precursor of the imported 58 kDa protein. Note that Ackerman et al. [5] showed that the product of *ATP13* (which has the same N-terminus as that of Aep2p) was found to be imported and processed. We suggest that cleavage of the 64 kDa product results in the removal of a targeting sequence about 6 kDa in length (approximately 50 amino acids). This is consistent with the cleavage position anticipated on inspection of the *AEP2* sequence where the positively charged, serine/threonine-rich region ends at about the amino acid residue 52. The data of Ackerman et al. [5] implied the shift in apparent size of the putative precursor of the mature product of *ATP13* to be from 42 kDa to 40 kDa, suggestive of cleavage of the N-terminus at approximately amino acid 20–25; however, the significance of these proteins remains open to question (see below). Note that in Fig. 3 there is only a trace of proteinase K-resistant material below the position of the 46 kDa marker, representing two minority species which we estimate at about 44 and 36 kDa in size. We conclude, therefore, that the size of Aep2p is about 64 kDa, which is processed to 58 kDa upon import into mitochondria. This is commensurate with the putative product of the *AEP2* ORF based on the sequence of this region of DNA [8].

The reported size of the *ATP13* protein product as 42 kDa is in disagreement with the present results. Ackerman et al. [5] used the hybrid-select procedure to capture mRNA from *S. cerevisiae* cells by hybridisation to immobilised cloned DNA containing *ATP13* sequences; the RNA was then released and translated in vitro in a rabbit reticulocyte lysate. In our work the RNA template for translation in the reticulocyte lysate was derived from in vitro transcription of pSP64T; the product RNA contains vertebrate globin sequences both 5′ and 3′ to the *AEP2* ORF. The discrepancy may arise if the natural yeast mRNA of Ackerman et al. [5] is not fully translated in vitro. Alternatively, nucleolytic degradation of mRNA or proteolytic degradation of the translation product may have generated the observed 42 kDa product.

The sequence differences between *ATP13* and *AEP2* are minimal; the only authenticated difference is at nucleotide 1012 (T in *AEP2*, C in *ATP13*: which generates amino acids Trp and Arg, respectively). This difference represents a common polymorphism in the *AEP2* gene with many laboratory strains having one or other amino acid at this position (L. Helfenbaum, T.P. Ellis, H.B. Lukins and P. Nagley, unpublished data) and it is not considered to generate a major mobility difference in the polypeptide.

The mitochondrial localisation of Aep2p is consistent with recent studies in our laboratory that suggest direct physical interaction between Aep2p and the 5′ untranslated region of *oli1* mRNA encoding subunit 9 of the ATP synthase complex (T.P. Ellis, L. Helfenbaum, B.E. Corner, H.B. Lukins and P. Nagley, unpublished data). In conclusion, we have shown that the entire *AEP2* ORF is a functional unit both genetically and biochemically. The present work provides great confidence that further studies on Aep2p will have a biological significance in the understanding of nuclear–mitochondrial interactions.

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